THE USE OF LASER-ASSISTED HATCHING IN BOVINE *IN VITRO* PRODUCED EMBRYOS TO IMPROVE PREGNANCY RATE

A Thesis

by

SUZANNE LYNN MENGES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2008

Major Subject: Physiology of Reproduction

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Approved by:

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Committee Members, Charles Long
Mark Westhusin
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ABSTRACT

The Use of Laser-Assisted Hatching in Bovine *in Vitro* Produced Embryos to Improve Pregnancy Rate. (August 2008)

Suzanne Lynn Menges, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Duane Kraemer

In vitro produced (IVP) embryos not hatching from the zona pellucida (ZP) after transfer is one possible contributing factor of a lower pregnancy rate when compared to *in vivo* embryos. This study evaluates using a microscope objective mounted laser to cut the ZP prior to transfer into the recipient to assist hatching. The preliminary data evaluated the effect of laser treatment on IVP embryos and subsequent blastomere survival. In six replicates, bovine oocytes were *in vitro* produced according to the standard laboratory procedures of TransOva Genetics, Sioux Center, IA. On days 5, 6, and 7 of *in vitro* culture, embryos were randomly divided into 3 groups: no treatment (Control; n=63), sham ZP cut (Sham; n=68), or ZP cut (Cut; n=70). Control embryos were immediately returned to the incubator. Sham embryos were exposed to all conditions as Cut except laser assisted hatching. The XyClone® system was used to treat the Cut group using pulse strength of 90% and pulse length of 600 µsec. Embryos were returned to culture until day 8 when embryonic development and the percentage of live cells were determined and analyzed with Chi square. The number of developing embryos

and the percentage of live cells per embryo showed no significant difference. Mean live cells ranged from 89-96% regardless of day of treatment. The laser assisted hatching effect on IVP embryo viability was evaluated by randomly dividing commercially produced embryos obtained from TransOva Genetics into two groups on day of transfer, Control or Cut. The ZP of treated embryos were cut with the laser using 80% pulse strength and pulse length of 500 µsec on day 7, immediately prior to transfer into estrous synchronized recipients. Ultrasonagraphy determined pregnancy rates. Thirty day pregnancy rates were 49.2% and 54.1% for Control (n= 189) and Cut (n=148) embryos, respectively, and were not statistically different ($p > 0.05$). However, 60 day Control pregnancy rate was 45.7% (n= 166) and the Cut group rate was 57.7% (n= 123) revealing a statistical difference ($p < 0.05$). These results demonstrate that the XyClone® system assisted hatching can improve 60 day pregnancy rates for IVP embryos by approximately 11 %.

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CHAPTER I

INTRODUCTION

In vitro fertilization can be a valuable instrument in the ability to assist genetic selection strategies and breeding plans for cattle production systems. It can be utilized to improve pregnancy rates in herds with low fertility or certain reproductive breakdowns such as ovulation and fertilization failures or reproductive tract blockage. *In vitro* fertilization allows for the more efficient use of semen preserved from deceased genetic lines or highly valuable semen. There is also the potential to amplify the use of small amounts of semen such as with sexed sperm. Although it is common to find some disadvantages in the *in vitro* fertilization system when compared to *in vivo* embryo production, research is ongoing to improve the technical aspects including the atmosphere for oocyte maturation, sperm treatment, fertilization factors, various embryo culture environments and recipient quality and synchrony (Hanson, 2006).

One of the greatest frustrations with *in vitro* embryo production is the loss of embryo development and subsequent pregnancy rate when utilizing *in vitro* culture (Gardner and Lane 2005). The American Embryo Transfer Association reported in 2005 that the average pregnancy rate for *in vivo* collected embryos transferred was 62.34% while the transfer pregnancy rate for the *in vitro* fertilized and produced embryos was reported at 43% (AETA 2005).

Success of pregnancy initiation in assisted reproduction depends on the relationship between the transferred embryo and the endometrium. The uterine environment of the estrous synchronized recipient animal must be conducive to accept

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This thesis follows the style of *Journal of Reproduction*.

the implantation of the hatched embryo that appears morphologically viable. The oocyte or embryo is surrounded by a cellular matrix called the zona pellucidae (ZP). The ZP provides several functions for the embryo, including protection against bacterial/viral/fungal infection and physical damage (Gordon 1994). The ZP also presents a species-specific sperm receptor and barrier that contributes to the prevention of polyspermy (Coy *et al*. 2008, Suzuki *et al.* 1994). As the embryo grows and develops, the blastocyst must hatch or escape the ZP that surrounds the cell mass to allow for the embryo to implant into the uterine wall for subsequent development. The first physicochemical event to allow for hatching is lysis of the ZP which is initiated by digestion with proteolytic enzymes produced by the embryo and/or female reproductive tract. The ZP lysis is not uniform but localized in one place (Flechon and Renard 1978). The second event which concludes hatching is the increase of hydrostatic pressure within the embryo exerted on the ZP caused by the expansion of the blastocoel cavity. In bovine the ZP is not profoundly changed during or after hatching (Flechon and Renard 1978). Implantation of the embryo can be obstructed for a few reasons such as embryonic structural abnormalities or imperfect endometrial receptivity but hatching failure can also occur (Gonzales *et al.* 1996). It is speculated that failure of the embryo to hatch following fertilization in vitro and subsequent transfer may be one of the reasons for the low efficiency of pregnancy (Vajta *et al*. 1997, Drobnis *et al*. 1988).

Failed hatching can be due to additional factors, one being the hardening of the zona pellucida. Zona hardening in human embryos has been related to multiple factors but primarily is associated with elevated levels of FSH and advanced maternal age (Loret *et al*. 1997; Cohen *et al*. 1992, Parikh *et al*. 1996, Meldrum *et al*. 1998). In the mouse

embryo, a suboptimal culture condition is attributed to this hardening effect (Dokras *et al*. 1991). Research has shown that partial digestion of the outer layer of the ZP had little impact on implantation rates, suggesting that the limiting factor of hatching is not due to ZP thickness but resilience of the thin inner layer or ZP hardening (Tucker *et al* 1993; Schiew *et al*. 1995; Al-Nuaim and Jenkins 2002). Other studies in human *in vitro* fertilization have shown a higher clinical pregnancy rate when there is a thinning of the ZP in which a complete incision or hole is not created (Mantoudis *et al*. 2001; Schmoll *et al*. 2003). Assisted hatching is based on creating artificial openings in the zona pellucida to promote the *in vivo* hatching progression. Assisted hatching can be achieved chemically with acid tyrode ZP drilling or pronase enzymatic digestion for ZP thinning. Hatching can be assisted mechanically through partial ZP dissection with a glass pipette, ZP drilling with piezo-micromanipulator and ZP drilling with a diode laser (Balaban *et al*. 2002, Cohen *et al*. 1992, Nakayama *et al*. 1998). Chemical dissolution of the ZP can decrease the number of intact blastomeres after hatching and have a negative effect on pregnancy rate when compared to mechanical assistance (Joris *et al*. 2003, Lanzendorf *et al*. 2007). The ZP partial dissection with a pipette also has drawbacks in the lack of producing reliable equally sized holes in the ZP. This dissection along with ZP drilling with the peizo require micromanipulation technical skills and are time-consuming leading to a detrimental amount of time out of the incubator or uterine environment (Al-Nuaim and Jenkins 2002).

The laser diode has several advantages over the other techniques as the laser beam is focused through the microscope objective to allow rapid, easy microdrilling with a high reproducibility (Makrakis *et al*. 2006). The IR laser radiation travels in the opposite

direction to the visible light and reaches a target in a similar manner to fluorescent illumination except for the radiation is concentrated on the target (Tadir and Douglas-Hamilton 2007). The beam diameter is generally 4.5-5 µm, which is smaller than the average thickness of the ZP at 14-18 µm (Tadir and Douglas-Hamiliton 2007). The size of the opening created can be regulated allowing for alternative approaches to the drilling depending on effect desired or potential of cell mass damage (Blake *et al*. 2001, Mantoudis *et al*. 2001). The area of the ZP around the focal beam of the laser is affected by heat conduction and thermal interaction (Tadir and Douglas Hamiliton 2007). It is extremely important to monitor pulse length and intensity in each laser setup and each laboratory environment to avoid damaging of the healthy cell mass (Douglas-Hamilton and Conia 2001). Studies have demonstrated when embryos are damaged by the laser that decreasing the laser beam intensity and increasing the number of pulses has allowed for the last increase in pregnancy rate (Tinnery *et al*. 2005). The laser diode procedure can be performed in conventional dishes with conventional media and does not require additional micromanipulation equipment.

Assisted hatching has proven through numerous studies performed on non-selected patients to be beneficial in the human *in vitro* embryo production model and increase clinical pregnancy rates and implantation rates (Edi-Osagie *et al*.2003, Seif *et al*. 2007, Liu *et al*. 1993, Meldrum *et al*. 1998, Hu *et al*. 1996). Assisted hatching has also been advantageous with cryopreserved embryos (Balaban *et al* 2006, Hiraoka *et al*. 2007). Patients with poor prognosis of pregnancy have also seen advantage with assisted hatching procedures (Schoolcraft *et al*. 1994, Antinori *et al* 1996, Obruca *et al*. 1994). There have been reports contesting this overall proven benefit and displaying that it is not

necessarily an answer to all repeated implantation failure (Tucker *et al*. 1996, Hellabaut *et al*. 1996). If infertility is due to suboptimal embryo quality, laser assisted hatching may not improve pregnancy rates (Grace *et al* 2007). It stands to reason if there is a potential benefit for assisted hatching in human or mouse in vitro models, bovine *in vitro* fertilization procedures could also benefit from this practice. The laser diode seems to be the most appropriate approach for commercial applications as it leads to less embryo damage and increased laboratory efficiency.

This experiment was designed to test the hypothesis that laser assisted hatching can improve the pregnancy rate of the bovine *in vitro* fertilization protocol. *In vitro* fertilization is valuable in the cattle reproduction industry. Assisted hatching might be developed to obtain a higher percentage of embryo implantation and therefore yield more calves for the producers. The first experiment determined the proper settings for the laser and the appropriate timing of the method to produce optimal embryonic development *in vitro*. The second experiment was a non-selective study to analyze pregnancy rate after the utilization of assisted hatching in the *in vitro* fertilization program. This was a comparative analysis against a control fresh transfer of *in vitro* produced embryos. The third experiment was designed to take certain characteristics of the embryos into consideration such as stage of development, embryo quality and whether the treatment was performed in a straw versus a dish. All pregnancy rates were determined at day 30 and day 60 of gestation. Together these data suggest with certain criteria such as superovulated donors, assisted hatching could increase the percentage of *in vitro* produced embryos that are capable of initiating a pregnancy in a cow.

CHAPTER II

MATERIALS AND METHODS

Media and Chemicals

Maturation medium consists of 0.00125 units per ml of FSH (Sioux Biochemical 715), 0.00125 units per ml of LH (Sioux Biochemical 725), 10 % fetal bovine serum (Hyclone SH30070.02), and 1% Penicillin streptomycin (Gibco 15140-122) and Medium 199 with Earles salts (Gibco 11150-042). . All T. L. Hepes (Biowhitaker 04-616F) used for washing or holding medium contained 1% Penicillin Streptomycin (Gibco 15140-122). Fertilization medium stock solution contained the following: 114 mM NaCl (Sigma S5886), 3.2 mM KCl (Sigma P5405), 25.0 mM $NaHCO_3$ (Sigma S5761), 0.34 mM $NaH_2PO_4H_2O$ (Sigma S9638), 10 mM Na Lactate (Sigma L7900), 1µl/ml Phenol Red (Sigma P0290), 2.0 mM $CaCl_22H_20$ (Sigma C7902), and 0.5 mM $MgCl_26H_20$ (Sigma M2393). Fertilization pyruvate stock consisted of 125 mM Na Pyruvate (Sigma P5280) and 0.9% saline. A solution for PHE is mixed with 20 µM Penicillamine, 10 µM Hypotaurine, 2 µM Epinephrine and 0.9% saline. Heparin aliquots used to supplement the fertilization medium were prepared at varied concentrations including; 0.005 mg/ml, 0.025 mg/ml, 0.050 mg/ml, 0.100 mg/ml, 0.150 mg/ml, 0.200 mg/ml, 0.250 mg/ml, 0.300 mg/ml, 0.400 mg/ml, and 0.500 mg/ml. The fertilization working solution consisted of fertilization stock solution, 10 µl per ml of pyruvate fertilization stock, 1% penicillin streptomycin, and 6 mg per ml BSA fraction V, Fatty Acid Free (Sigma A6003). All media were prepared through sterile techniques and filtered through a 0.20 µm filter.

Transova culture medium is a proprietary medium made by Transova's IVF lab in Sioux Center, Iowa.

Recipient Synchronization

The estrous cycle of all recipient cattle were synchronized. A CIDR implant (Pfizer) was vaginally inserted and 2 cc of GNRH (Ovacyst) was administered intramuscularly on day 0 of the synchronization schedule. On the PM of day 6, the CIDR implant was removed and 6cc of Prostaglandin (Lutalyse, Pfizer) or (Prostamate, Agri-Labs) was intramuscularly injected. The cattle were expected to demonstrate signs of estrus starting 36 hours later. All estrus signs were observed and recorded. Typically recipients exhibiting estrus in the range of 48 hours prior to and 36 hours following aspiration of the oocytes used for *in vitro* fertilization were utilized for embryo transfer on day 7 post fertilization.

Experiment 1

Oocyte Maturation

Embryos were produced by *in vitro* maturation and fertilization of bovine oocytes purchased through Concho Valley Genetics (San Angelo, Texas, USA). Oocytes were aspirated from slaughter house ovaries into T.L. Hepes holding medium with 0.02673 g/ 500 mls Heparin (Sigma H3393) added. Only oocytes classified as grade A and grade B were selected and washed into a 35mm petri dish filled with 3mls of T.L. Hepes holding

medium. Grade A and B oocytes possess two or more layers of cumulus cells and have an even colored healthy cytoplasm with a strong cytoplasmic membrane. Approximately 50 to 60 selected oocytes were washed through two 35 mm petri dishes of T. L. Hepes holding medium before being transferred into a glass vial filled with 1ml of maturation medium as described above.. The medium was pre-equilibrated in humidified 5% CO_2 and air. The vials containing the oocytes were transported to the lab in incubators maintained at 38.5ºC and 90% of the oocytes arrived at the metaphase II stage of meiosis with the first polar body extruded.

In Vitro *Fertilization and* In Vitro *Culture*

Fertilization working medium was prepared and equilibrated for 2 hours in 5% CO2 and humified air atmosphere. A 4 well Nunc dish was prepared with 425ul of fertilization medium in each well. At 22 hours post maturation, oocytes were removed from the shipping vials and washed through two 35mm petri dishes containing 3mls of T.L. Hepes holding medium. Oocytes were then washed through one well of fertilization medium before being placed into the well to be fertilized. All dishes containing oocytes were then placed back into the incubators as soon as possible. Frozen semen was thawed in a 38.5ºC water bath and live sperm cells were separated using an Isolate (Irvine Scientific 99264) gradient as directed by the product insert. The gradient was spun in a centrifuge at 800 rpm and then the sperm pellet at the bottom of the gradient was separated and counted. Oocytes were fertilized using 2 million sperm cells per ml of media and 20 µg per ml of Heparin. Each fertilization well also received 20 µl of the

PHE stock solution. Oocytes and sperm were allowed to incubate at 38.5ºC in 5% CO2 humified air for 18 to 20 hours. At this point, embryos were removed from the fertilization wells and placed into 0.5 ml of TL Hepes holding medium in a 15 ml conical tube. The tube was placed on a vortex at low speed for 1.5 minutes and then washed with 4ml of T.L. Hepes holding medium into a 60mm petri dish. All embryos were washed through 3mls of T.L. Hepes in a 35mm petri dish. Before being placed into culture, embryos were washed through 500µl of G1 medium (Vitrolife, Englewood, CO, USA, 10127) with 8µg/ml of BSA (Probumin, Millipore, 81-068-3) that had been pre-equilibrated in 5% CO2, 5% O2 and humidified air at 38.5 C. The embryos were finally placed into the culture well of 500µl G1 medium under mineral oil for 72 hours. After the 72 hours, embryos were washed through one 500 µl well of G2 culture medium (Vitrolife, Englewood, CO, USA, 10131) and transferred in a final 500 µl well of G2 culture media under mineral oil until day 7 when the embryos are ready to transfer.

Embryo Treatments

Viable embryos were obtained from 6 different replicates of *in vitro* production. With each individual IVF production, embryos were removed from culture on either day 5, day 6, or day 7 and placed into a 35mm petri dish of 3mls of Vigro Hold medium (Bioniche, Pullman, WA, USA) to be evaluated. All grade 1 and 2 embryos at the appropriate stage of development were utilized and divided into three groups with even distribution of grades and stages. The first group was considered the control set and was immediately washed back into a 500 µl well of pre-equilibrated G2 medium in 5% CO2,

5% O2, and air at 38.5 C. A second set of embryos was assigned to the sham ZP cut group and placed into a separate well containing 500 µls of Vigro Holding medium in the same 4 well nunc dish as the ZP of the embryos being pierced with the laser. The third set of embryos was treated with the Hamiliton Thorne XYClone Laser Ablator and classified as ZP cut.

The XYClone Laser is a Class 1 laser incorporated into a 40X objective that installs directly on the turret of the inverted microscope. The 300 milliwatt laser emits a 3.5 µm diameter beam at a wavelength of 1480 nm. In preliminary studies we tested a pulse strength of 90% at a pulse length of 1850 µs. This treatment proved to be detrimental by destroying a high percentage of live cells in the embryo cell mass. We therefore reduced the pulse length to 600 µs and 90% power for all subsequent studies. The treated group of embryos was placed into a 4 well nunc dish with 500 µl of holding medium and placed on the heated stage of an inverted microscope. Each embryo zona pellucida was centered through the objective and under the unique Isotherm Rings of the XYClone Standard Software. A hole was produced in the zona of each embryo as quickly as possible. If possible, the hole was drilled in an area of the zona that was closest to the largest perivitelline space. This was done to avoid any harmful effects of the heat from the laser on the cell mass of the embryo. If the embryo was at a more advanced stage of development, a complete hole was not always cut to avoid damage to the developing trophectoderm. Once all embryos were cut, the Sham group and Cut group were washed through one well of 500 µls pre-equilibrated G2 medium in 5% Co2, 5%O2 and humidified air at 38.5 C before being placed into the final 500 µl culture well.

All embryos from each treatment group were analyzed on day 8 of culture. All three groups were washed into 500 µls of Vigro Hold (Bioniche, Pullman, WA, USA) or TL Hepes holding medium warmed to 38.5ºC. All stages and grades from each group and each replicate were recorded. The total number of nuclei per embryo and the membrane damage to the nuclei was analyzed by live/dead stain. Embryos were held in a 4-well Nunc dish with a 500 µl drop of Vigro Hold or T. L. Hepes containing 2.5 µg/ml of Hoechst 33342 (Sigma H6024) and 1.0 mg/ml of Propidium Iodide (Sigma P4864) for a total of 15 minutes @ 37ºC. An additional wash through 500 µls of Vigro Hold or T.L. Hepes was performed before the embryos are mounted on a glass slide. Each group of embryos was mounted five per slide and moved into a 25µl drop of PBS before being secured with a cover slip. Embryos are examined using fluorescent microscopy and all live cells were counted along with all damaged cells for each embryo. All data were recorded and used for statistical analysis.

Experiment 2

Oocyte Maturation

Embryo production was performed according to standard operating procedures of Transova Genetics, Sioux Center, IA. Oocytes were collected from ovaries obtained from the slaughter house. Follicles between 2mm and 8 mm in size were aspirated with a 18 g needle. Oocytes of the same quality as mentioned before were selected and washed and described in Experiment 1. Maturation medium was pre-equilibrated in humidified

5% CO_2 and air in 4 well nunc dishes. Each well contained 500 µls of maturation medium. Approximately 50 oocytes were washed through one well of maturation medium before being placed into a final culture well. All dishes were returned to the humified 5% CO_2 and air atmosphere.

In Vitro *Fertilization and* In Vitro *Culture*

The following day fertilization working medium was prepared containing 20 µg per ml of Heparin and 40 µl per ml of the PHE working solution. This medium was aliquoted into 500 µl drops in a 6 well dish pre-equilibrated at 5% CO_2 and air for at least two hours. At 20 to 22 hours post maturation, oocytes were washed through two 35 mm petri dishes containing 2-3 mls of T.L Hepes holding solution and then washed through two wells of the pre-eqiulibrated fertilization medium before being placed into the final drop for fertilization. Oocytes were placed back into the incubator as soon as possible. Frozen sexed semen was thawed in a 38.5ºC water bath and live sperm cells were separated using an Isolate (Irvine Scientific 99264) gradient as directed by the product insert. The gradient was spun in a centrifuge at 600 rpm for 15 minutes. The sperm pellet at the bottom of the gradient was separated and placed into another 15 ml conical tube with 1 ml of fertilization medium. This dilution was spun in the centrifuge at 400 rpm for 5 minutes to wash the semen free of potential contaminates. The sperm pellet was then separated once again and semen was added to the fertilization well containing the oocytes at an approximate concentration of 2 million sperm cells per ml of medium. Semen was not counted, it was only added by approximation from the laboratory

technician. Oocytes and sperm were allowed to incubate at 38.5ºC in 5% CO2 humified air for 18 to 20 hours. A 6 well dish of Transova culture medium was prepared with 500 µls of culture medium in each well and pre-equilibrated in a humified 5% CO_2, 6% O_2, and air atmosphere. At this point, embryos were removed from the fertilization wells and placed into 3 mls of TL Hepes holding medium in a 35mm petri dish. Cumulus cells were removed by repeat pipetting and oocytes were washed through an additional 3 mls of T. L. Hepes washing medium in a 35 mm petri dish. Oocytes were then washed through two wells of culture medium before being placed into the final culture drop.

Embryo Treatments

Seven days later the dishes were removed from the incubator and embryos were moved into a 4 well nunc dish containing 500 µl drops of Vigro Holding medium (Bioniche EVM024). Only embryos classified as grade 1 or a high grade 2 were utilized. Embryos were equally divided into a control group and a treated group. The treated group was placed on the microscope affixed with the XYClone Laser. The setting was set at a 600 µsec pulse length at 90% power. A hole was drilled in the zona pellucida of each embryo as described above for experiment 1. All embryos were then loaded into a 0.25 cc straw. Straws were loaded into an embryo transfer gun (IMV 007239) and covered by a blue embryo transfer sheath (IMV 006385) and a sanitary sleeve (IMV 006458). Embryos were then non surgically transferred through vaginal insertion into the uterine horn of the estrus synchronized recipient that corresponded to the side of the tract that contained a corpus luteum in the ovary. Stage and grade of embryo were not

recorded. Pregnancy rate was initially determined on day 30 and then verified at day 60 of gestation through ultrasound.

Experiment 3

Donor Syncronization and Aspiration

All in-vitro embryo production and oocyte retrieval was performed by Transova Genetics either in the Texas center in Bryan, TX or in the Iowa center in Sioux Center, Iowa. Donor cattle were ultrasounded before being set up for aspiration. Various synchronization protocols were utilized depending on the need of the donor. Some donors received a CIDR (Pfizer Animal Health) with 2cc of Ovacyst (Webster Veterinary Supply, 07-839-1830) on what is considered Day 0. Other donors received a dominant follicule removal, DFR, through an ultrasound guided transvaginal probe. If a CIDR was implanted then on in the PM of day 4 of the protocol the first Folltropin (Bioniche) shot was administered. The second Folltropin was given in the AM of day 5 and the third in the AM of day 6. Oocyte aspiration took place on day 7 of this protocol. If a DFR was performed on day 0, the first Folltropin shot was given in the PM of day 1, with subsequent shots given the in the AM of day 2 and day 3. On day 4, oocyte removal was performed. Oocytes were collected from donor cattle through transvaginal ultrasound guided ovum pick-up procedures. An 18 gauge aspiration needle was rinsed with TL Hepes holding medium before being inserted into the guide in the vaginal probe. The needle was connected through tubing to either a Sure Flush embryo filter (PETS) or an

Emcon filter (PETS) and a vacuum pump. Using the ultrasound as a guide, only follicles that were between 2 mm and 8 mm in diameter were aspirated. Follicular fluid and oocytes were removed from the follicles and aspirated into the filter. All oocytes including denuded and expanded oocytes were washed into 3 mls of TL Hepes holding medium in a 35mm petri dish.

Oocyte Maturation

Maturation medium was prepared equilibrated as described for experiment 2. All media for maturation, fertilization and culture were prepared as microdrops. If 1-10 oocytes were obtained then 50 µl of medium was utilized. If 11-20 oocytes were aspirated then 100 µl of medium was used and 200 µl drops were used if 21-40 oocytes are acquired. All drops were covered in embryo tested mineral oil (Sigma M8410). Six well plates were used for all maturation, fertilization, and culture and two wash wells of 200 µl of medium were prepared for each donors. All oocytes were washed through two drops of maturation medium before being matured in a final drop of pre-equilibrated maturation medium. All donors oocytes were kept separate and were fertilized individually.

In Vitro *Fertilization and* In Vitro *Culture*

Fertilization medium was prepared and equilibrated as described for experiment 2. Frozen semen was separated using an Isolate (Irvine Scientific 99264) as directed by

the product insert. The gradient was spun in a centrifuge at 600 rpm for 15 minutes. The sperm pellet at the bottom of the gradient was separated and placed into another 15 ml conical tube with 1 ml of fertilization medium. This dilution was spun in the centrifuge at 400 rpm for 5 minutes to wash the semen of potential contaminates. The sperm pellet was then separated once again and semen was added to the fertilization well containing the oocytes at an approximate concentration of 2 million sperm cells per ml of medium. Semen was not counted, it was added by approximation from the laboratory technician. Transova culture medium was pre-equilibrated in 5% CO2, 5% O2 and humified air at 38.5 ºC in microdrops. All oocytes were denuded at 18 to 22 hours post fertilization through agitation with a micropipettor in a 35 mm petri dish containing TL Hepes holding medium. Once all cumulus cells were removed, the embryos were washed through two 50 µls drops of Transova culture medium and then transferred to the final well of culture medium. Embryos are cultured for seven days before being transferred into an estrous synchronized recipient cow.

Embryo Treatment

On day 7, embryos are removed from culture medium and washed through Vigro Hold medium (Bioniche EVM024) warmed to 38.5ºC. Embryos were equally distributed from each donor and sire mating into a control group and a treated group. The treated group was placed on the microscope affixed with the XYClone Laser. Some replicates were cut with the laser while still in the dish while other replicates were cut after already being loaded in a 0.25cc straw. The setting was set at a 600 µsec pulse length at 90%

power for the embryos being treated at the center in Bryan, TX. The settings were lowered to 500 μsec pulse length at 80% power for the treatments in Sioux Center, IA. A hole was drilled in the zona pellucida of each embryo as described above for experiment 1. All embryos were then loaded into a 0.25 cc straw and transferred into estrous synchronized recipients as explained above in experiment 2. Donor identification, stage of embryo development, grade of embryo, and location of embryo when being cut were all recorded. Pregnancy rate was initially determined on day 30 and then verified at day 60 of gestation through ultrasound.

Statistical Analysis

In experiment 1, 6 replicates containing a total of 229 embryos were tested and analyzed. Experiment 2 consisted of 3 replicates of 112 embryos and experiment 3 contained 337 embryos tested for pregnancy at day 30 and 280 embryos ultrasounded on day 60. Chi-square was utilized to statistically analyze all data.

CHAPTER III

RESULTS

Experiment 1

The original laser power of 90% with a laser duration length of 1850 µsec proved to be very detrimental to the survival of embryos. The 28 embryos that were cut with this laser strength and power on day 5 averaged 70% live embryonic cells at day 8 of culture. It was decided to shorten the laser length time to 600 µsec maintaining the power at 90%. The day on which the zona pellucida was cut at these settings to assist hatching did not seem to alter the mean of the percentage of live cells (Table 1). The variation of the mean of viable cells ranged from 84.4% to 96% (Table 1).

Table 1. Comparison of assisted hatching with mean percentage of viable cells in embryos on day 8 of *in vitro* culture.

	Day 5 (n)	**Day 6 (n)**	**Day 7 (n)**
Cut	92.8 ±4.7 (5)	91.9 ±2.3 (43)	92.9 ±1.7 (22)
Sham	84.4 ±3.5 (32)	92.9 ±1.4 (42)	89.8 ±1.5 (22)
Control	96 ±1.1 (8)	89.9 ±3.1 (31)	93.2 ±1.1 (24)

There was no significant difference.

There was some variability in the percentage of embryos that had either started hatching or where hatched on day 8 of culture when analyzed. Yet with all sets of

embryos cut on each day of treatment, the cut group had a greater percentage of embryos that had initiated hatching or already hatched through the zona pellucida (Table 2).

Table 2. Comparison of assisted hatching with percentage of hatching or hatched embryos on day 8 of *in vitro* culture.

	Day 5 (n)	**Day 6 (n)**	**Day 7 (n)**
Cut	**60.0 % (5)**	**67.4 % (43)**	**54.5 % (22)**
Sham	**34.4 % (32)**	**54.8 % (42)**	**22.7 % (22)**
Control	**50.0 % (8)**	**25.8 % (31)**	**50.0 % (24)**

Experiment 2

Through 3 replicates of in vitro fertilization with slaughter house derived oocytes, there were 112 viable embryos that resulted on day 7. There were a total of 59 embryos that were treated with the ZY Clone laser. This resulted in a 50.8% pregnancy rate (Table 3). The 53 control embryos transferred produced a 49.0% pregnancy rate (Table 3). There was no statistical significance demonstrated with these results.

Table 3. Pregnancy rate comparison of assisted hatching obtained from embryos derived from slaughter house donors through *in vitro* fertilization with sexed semen at day 60 of gestation.

Treatment Group	Replicate	# Transferred	# Pregnant	% Pregnant
Cut	1	21	13	61.0 %
Cut	2	16	6	37.5 %
Cut	3	22	11	50.0 %
Cut	**Sub Total**	**59**	**30**	**50.8 %**
Control	1	16	9	56.3 %
Control	2	17	6	35.3 %
Control	3	20	11	55.0 %
Control	**Sub Total**	**53**	**26**	**49.0 %**

*There was no significant difference.

Experiment 3

Although the overall pregnancy rate on day 30 from both locations was higher when the hatching was assisted on day 7 of culture before transfer, the differences were not statistically significant (p-value = 0.377). Eighty embryos out of a total of 148 went on to fetal development after laser assisted hatching and subsequent transfer. Of the 189 controls transferred, 93 pregnancies were detected at day 30. This produced an increase of 4.84% in pregnancy rate when the laser was used to rupture the ZP of the embryos but was not statistically significant (Table 4).

Table 4. Pregnancy rate at day 30 of gestation obtained from *in vitro* fertilization of commercial donors after use of assisted hatching.

Treatment	# Transferred	# Pregnant	% Pregnant
Cut	148	80	54.1 %
Control	189	93	49.2 %

*No significant difference.

The 60 day pregnancy rate did show a significant increase in pregnancies obtained with the treatment of assisted hatching with a laser. Due to commercial company conditions, not all pregnancies were monitored at day 60. There were a total of 280 recipients that were checked for pregnancy by ultrasound at day 60 of gestation. There were 123 treated embryos in which the zona pellucida was cut with the laser. Of these embryos, 71 went on to initiate a pregnancy. This was an 11.86% increase over the control embryo transfers from 72 pregnant out of 157 embryos transferred. This increase in pregnancy was statistically significant (Table 5).

Table 5. Pregnancy rate at day 60 of gestation obtained from *in vitro* fertilization on commercial donors after the use of assisted hatching.

	# Transferred	# Pregnant	% Pregnant
Cut	123	71	57.7 %
Control	157	72	45.9 %

*Values within this column are statistically different $P < 0.05$.

The embryos that were transferred in the Texas Transova location showed a 27.38% decrease in pregnancy rate when a laser was used to assist hatching (Table 6). With a total of 57 embryos transferred, this is a small subset of the complete data. These embryos were transported to another facility to utilize the XY Clone laser unit. This dramatic loss of pregnancy could be due to transportation effects that were not specifically tested. These procedures were also performed in the summer months in Texas therefore the transportation of these embryos could have been exposed to highly unfavorable temperatures and conditions. This result was statistically significant.

Table 6. Pregnancy rate at day 30 of gestation obtained from commercial donors at the Texas Transova location after the use of laser assisted hatching.

	# Transferred	**# Pregnant**	**% Pregnant**
Cut	25	8	32.0 %
Control	32	19	59.4 %

*Values within this column are statistically different P <0.05.

There were a total of 280 embryos transferred at the Transova center in Sioux Center, Iowa. The thirty day pregnancy exam revealed 58.54% pregnancy rate when utilizing laser assisted hatching compared to 47.13% pregnant in the control transfers. Although there was an 11.41% increase in pregnancy this did not prove to be statistically significant with a p-value of 0.0576. There were two pregnancies lost in the control group and one pregnancy lost in the treated group between the 30 day ultrasound exam and the 60 day ultrasound exam. This resulted in an 11.86% increase of pregnancy with the laser assisted hatching at day 60 of gestation which did demonstrate a significant statistical difference with a p-value of 0.0484 (Table7).

Table 7. Pregnancy rate at day 30 and day 60 of gestation obtained from commercial donors at the Iowa Transova location after the use of laser assisted hatching.

	# Transferred	**# Pregnant 30 Day (%)**	**# Pregnant 60 Day (%)**
Cut	123	72 (58.5 %)	71 (57.7%)
Control	157	74 (47.1 %)	72 (45.9 %)

*Values within this column are statistically different P <0.05.

The zona pellucidae of 65 embryos were cut with the laser while still in a dish which produced 39 pregnancies. This was an increase of 3.86% over the 56.14% pregnancy rate when the embryos where loaded into a 0.25cc straw before being cut by the laser. Of the total of 57 embryos, 32 implanted to achieve a pregnancy using the straw as the vessel for treatment (Table 8). This was not statistically significant.

Table 8. Comparison of pregnancy rate between different vessels containing embryos during assisted hatching with the laser.

	# Transferred	**# Pregnant on Day 30 (%)**	**# Pregnant on Day 60 (%)**
Straw	**57**	**32 (56.1 %)**	**32 (56.1 %)**
Dish	**65**	**40 (61.5 %)**	**39 (60.0 %)**

*No significant difference.

The data were analyzed to compare the use of assisted hatching against the fresh transfer control within each stage of embryo to observe any benefit within any specific

stage of embryo development. The IETS has set standards for labeling the maturity of an embryo. Stage 4 is a compact morula, stage 5 is an early blastocyst, stage 6 is a full blastocyst, and stage 7 is a full blastocyst that has a thinned zona but has not had any lysis of the zona for hatching yet. The stage 4, stage 5 and stage 6 embryos showed an increase in pregnancy of 11.04%, 14.48%, and 22.67%, respectively. The stage 7 embryo had an 8.95% decrease in pregnancy rate but his was only out of a total of 10 treated embryos and 19 control embryos (Table 9). Stage 8 embryos were already hatching therefore no laser treatment was performed. There was no statistical difference throughout any of the stages of embryos.

Table 9. Comparison of pregnancy rate between different embryo stages with laser assisted hatching.

	% Pregnant Control (N)	**% Pregnant Assisted Hatched (N)**
Stage 4	39.0 % (30)	50.0 % (31)
Stage 5	48.5 % (16)	63.0 % (17)
Stage 6	44.0 % (11)	66.7 % (16)
Stage 7	79.0 % (15)	70.0 % (7)

*No significant difference.

Data were analyzed to investigate if assisted hatching could help improve pregnancy rate with each grade of embryo. There was a 13.1% improvement with grade 1 embryos with a p-value of 0.2115. Assisted hatching with the laser enhanced the pregnancy rate with grade 2 embryos by 13.2% (p-value equals 0.1698) and grade 3

embryos by 8.44% (p-value equals 0.4464) (Table 10). These results were not statistically significant.

Table 10. Comparison of 60 day pregnancy rate between different embryo grades when using laser assisted hatching.

	% Pregnant Control	**% Pregnant Assisted Hatched**
Grade 1	55.7 % (29)	68.2 % (30)
Grade 2	47.6 % (30)	60.9 % (28)
Grade 3	31.0 % (13)	39.4 % (13)

*No significant difference.

CHAPTER IV

DISCUSSION

The bovine embryo transfer industry utilizes *in vitro* embryo production for many reasons yet the resulting pregnancy rate averages lower than *in vivo* produced embryos (Rizos *et al*. 2002). Multiple factors contribute to this decrease of embryo implantation including embryo quality and hatching ability of the blastocyst. The human in-vitro production industry has utilized assisted hatching techniques to help improve conception rates in various types of patients and through various methods (Edi-Osagie *et al*. 2003, Seif *et al*. 2007, Liu *et al*. 1993, Meldrum *et al*. 1998, Hu *et al*. 1996). The XY Clone laser has proven to be the most efficient method and most limiting in cellular damage to the embryo. Poor prognosis patients, older patients and FSH stimulated patients have all shown improved pregnancy rates with the use of assisted hatching (Loret *et al*. 1997; Cohen *et al*. 1992, Parikh *et al*. 1996, Meldrum *et al*.1998).

The XY Clone laser was first evaluated for detrimental effects on the bovine in-vitro produced embryo. The original setting of a pulse length of 1850 µsec at a 90% power proved to cause too much cellular damage. The pulse length was reduced to 900 µsec and maintained at 90% power to cut a hole in the ZP of each embryo. It seems logical that the perivitelline space would be larger earlier in development therefore decreasing the opportunity for lysis of cells in the cell mass or blastoceol after utilization of the laser. Yet, there was no difference in hatching results and cellular division between days 5 to 7 on which the ZP was cut. There was an increase in the percentage of embryos that reached the hatching stage or fully hatched out of the ZP on day 8 of in vitro culture in the laser treated groups across all days of treatment. This increase would

be expected with a hole being punctured in the ZP. This continuation of embryo development and hatching combined no significant difference in viable cell percentage in the laser treated groups as compared with the control group would suggest that little harm is done when embryos are assisted hatched with the XY Clone laser. Also, by waiting until the day of the embryo implant on day 7 of culture, the embryo doesn't have to be removed from the incubator environment multiple times. This also lends to less time needed by the lab technician to perform the procedure.

The first series of transfers were performed with high quality embryos from slaughter house donors that were not stimulated with FSH. There was only one sire used across all three of the replicates. There was no significant increase in the conception rate with the use of the laser assisted hatching. The second series of transfers included a variety of donor, sires, embryo stages and grades. There were two different locations that performed the laser assisted hatching. One small set of embryos was assisted hatched with the laser in the Transova Texas center and demonstrated low pregnancy rates when compared to the controls. The laser performing the treatment was in a different facility as the incubators maintaining the embryo culture and as the recipients synchronized to receive them. The transportation with the high temperature from the middle of summer could have played a harmful role in further development.

A larger subset of embryos was analyzed at Transova in Sioux Center, Iowa. The assisted hatching treatment resulted in an increase in pregnancy rate of 11.86% at day 60 of gestation which proved to be statistically significant. At day 30 of gestation, the ultrasound exam identified an 11.41% increase in pregnancy that was not statistically significant. Two pregnancies were lost in the control group and one pregnancy was lost

in the treated group between day 30 and day 60 of gestation to shift the results into significance. The p-value for the 30 day pregnancy rate was 0.0576 and the p-value for the 60 day pregnancy rate was 0.0484. We considered statistical significance with a p-value of 0.05 or smaller. Since our result was so close to this value it is necessary to do additional research to increase the sample size to confidently declare this result as statistically significant. In comparing no difference of pregnancy rate in experiment 2 to an increase of pregnancy percentage in experiment 3, it is interesting to note the lack of follicle stimulation and the probability of the oocytes origin being from younger donors in experiment 2. It has been suggested in human in vitro embryo production that FSH stimulation and older women gain greater benefit from assisted hatching due to zona hardening (Loret *et al*. 1997; Cohen *et al*. 1992, Parikh *et al*. 1996, Meldrum *et al*. 1998). The oocytes in experiment 2 were derived from slaughter house donors that were more than likely younger females. The oocytes in experiment 3 were derived from donors in a commercial embryo transfer program that received FSH stimulation. A lot of donors that commercial producers decide to perform in vitro fertilization on are older donors that don't have a chance of reproduction through more conventional means. The comparison between experiment 2 and experiment 3 follow the theory set forth in human in vitro embryo production that older donors and hormone stimulated donors could benefit from laser assisted hatching.

There was an increase of pregnancy with assisted hatching in the stage 4, stage 5, and stage 6 embryos of 11.04%, 14.48%, and 22.67%, respectively. There was an 8.95% decrease of pregnancy in the stage 7 embryos. Although none of these results showed statistical significance, the decrease of stage 7 could be contributed to a thinned ZP and a

decrease perivitelline space that would cause a thermal interaction that would injure any cells near the site of incision. There was an increase of pregnancy rate after use of the laser displayed within each grade of embryo. Grade 1 embryo pregnancy rate was increased by 13.1%, grade 2 embryo pregnancy rate was increased by 13.2%, and grade 3 embryos pregnancy rate was increased by 8.44%. None of these results prove to be statistically significant. Yet an increase in pregnancy percentage resulted from the use of assisted hatching. Further research is needed to determine if an increase of pregnancy continues when a larger number of embryos are treated.

CHAPTER V

CONCLUSION

Laser assisted hatching can potentially have a positive effect on sixty day pregnancy outcome after the transfer of in-vitro fertilized embryos. A larger subset of embryos needs to be tested to appropriately analyze the effect of the grade of the embryo, the stage of the embryo and the vessel containing the embryo when the zona is being punctured.

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